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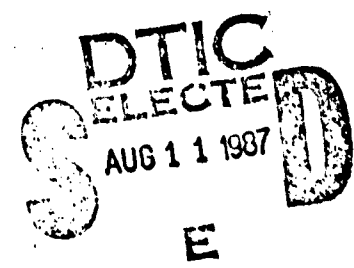
INDUCTION OF COLONY STIMULATING FACTOR IN VIVO BY RECOMBINANT INTERLEUKIN 1α AND RECOMBINANT TUMOR NECROSIS FACTOR α[1]

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In response to a potent inflammatory challenge, such as Gram-negative endotoxin, a number of cytokines are induced that, in turn, mediate many of the pathophysiologic alterations associated with endotoxicity. In this study, we have observed two endotoxin-associated monokines, recombinant interleukin-1α (rIL 1α) and recombinant tumor necrosis factor α (rTNFα), to induce colony stimulating factor (CSF) in vivo. The CSF activities produced in response to rIL 1α or rTNFα gave rise to a mixture of granulocyte-macrophage colonies and were induced in a dose- and time-dependent fashion. peaking within 3 hr of cytokine injection (preceding peak CSF induction by endotoxin by several hours). Combined injection of suboptimal concentrations of rIL 1α and rTNFα were additive, and simultaneous injection of optimal concentrations of each failed to increase CSF levels over that observed with either cytokine alone. Unlike endotoxin, neither cytokine induced interferon in vivo. These findings extend our understanding of the cytokine cascade that is operative in an inflammatory response and may account for many of the observed hematopoietic alterations that accompany inflammation.

Many of the pathophysiologic responses to endotoxin. the lipopolysaccharide (LPS) derived from Gram-negative bacteria. are mediated by the induction of specific macrophage-derived soluble factors (1-3). For example. LPS induces the production of interleukin 1 (IL 1). which in turn mediates fever. neutrophilia. hypoferremia. and the production of acute-phase reactants. such as fibrinogen. C-reactive protein. and serum amyloid A (4, 5). More recently. IL 1 has been reported to mimic the radioprotective effects of LPS (6). Tumor necrosis factor (TNF) has also been implicated as a mediator of endotoxicity by the demonstration that passive administration of polyclonal anti-TNF antibodies protected mice against lethal challenge with LPS (7). Both of these factors. as well as others associated with LPS-responsiveness. e.g.. glucocorticoid antagonizing factor (8) and interferon (IFN) (9). are present in maximal titers in the serum within several hours of LPS administration (10-12).

Recent evaluation of the effects of cloned. recombinant IL 1α (rIL 1α)[2] and recombinant TNFα (rTNFα) preparations has revealed that the induction sequence for these two factors. as well as the specific contribution each makes physiologically. may be more interdependent than originally imagined. Dinarello et al. (13) and Bachwich et al. (14) have shown that rTNFα induces IL 1 in macrophage cultures. Dinarello et al. (13) further showed that rTNFα itself was pyrogenic. and at high concentrations resulted in two cycles of fever: the first one TNF-mediated: the second. IL 1-mediated.

Colony stimulating factor (CSF) is also induced by LPS: however. serum levels of CSF are not maximal until approximately 6 hr after LPS administration (15). Before the availability of rIL 1. Kampschmidt. Upchurch. and Pullam (16, 17) provided the first evidence that CSF could be produced indirectly. in response to partially purified preparations of "leukocyte endogenous mediator." which were extracted from glycogen-induced peritoneal exudates. The findings presented here confirm and extend those of Kampschmidt and his co-workers. Both murine and human rIL 1α preparations. as well as rTNFα. induced in vivo serum CSF activity in a dose- and time-dependent fashion. which preceded peak induction of CSF by LPS. However. unlike LPS. neither cytokine induced IFN in vivo.

MATERIALS AND METHODS

Mice. For most of the experiments presented. C57BL/6J (The Jackson Laboratory. Bar Harbor. ME) mice were utilized. In addition. C3H/HeJ and C3H/OuJ mice (The Jackson Laboratory). C3H/HeN mice (Animal Genetics and Production Branch. NCI. Frederick. MD). and outbred HSD(ICR)BR mice (Harlan Sprague Dawley. Indianapolis. IN) were also used. Female mice. 6 to 12 wk of age. were used for all experiments. Mice were housed in cages with Micro-Isolation unit tops and were allowed access to food and acid water ad libitum.

Reagents. Murine rIL 1α (18) was obtained from Hoffmann-La-Roche. Inc. (Nutley. NJ: Ro 23-7390. Lot No. 14430-110-48) The murine rIL 1 was provided in 5 M guanidine hydrochloride (Gu-HCl) as a stabilizing agent. A control bacterial extract in Gu-HCl (No. 13146-129-49) was also supplied by Hoffmann-LaRoche. Inc. Human rIL 1α (19). provided in sucrose (as a stabilizing agent). was obtained from Immunex (Seattle. WA). At this time. there is no "international standard" for IL 1α preparations. and assignment of

[3] Abbreviations used in this paper: rIL 1α. recombinant interleukin 1-α. rTNFα. recombinant tumor necrosis factor α. CSF. colony stimulating factor: Gu-HCl. guanidinium hydrochloride.

"units" can vary from laboratory to laboratory. Therefore. because the murine and human reagents were obtained from different sources. it was essential to ensure comparability of units. To do this. both murine and human rIL 1α preparations were assayed simultaneously in a thymocyte proliferation assay (20). Briefly. serial two-fold dilutions of the rIL α preparations were made in bioassay medium (RPMI 1640 supplemented with 2 mM glutamine. 2F μM 2-mercaptoethanol. 100 U/ml penicillin and 100 μg/ml streptomycin. and 10% fetal calf serum (FCS)). To each set of triplicate wells of a 96-well plate (Falcon. Oxnard. CA) were added 50 μl/well of each dilution of rIL 1α and 50 μl/well of phytohemagglutinin (Enzo Biochemicals. NY: prepared in bioassay medium at a concentration of 1 μg/ml). Single cell thymocyte suspensions were prepared from 4- to 6-wk-old C3H/HeJ mice in bioassay media at 1.5×10^6 cells/ml. and 100 μl/well were added immediately upon preparation of the cell suspension. Cultures were incubated at 37°C. 5% CO_2 for 72 hr. pulsed with [³H]thymidine during the last 4 to 6 hr of incubation. and were harvested onto filters with a cell harvester (Brandel. Gaithersburg. MD). To determine relative units of activity. the scintillation data was plotted as log (counts per minute) vs log dilution⁻¹ and linear regression analysis was carried out over the linear portion of the dilution curve. The dilution at which half-maximal uptake was observed was extrapolated from the equation for the best line of fit and was assigned a value of one unit of activity. Based on the results of three separate thymocyte proliferation assays. we determined the murine and human rIL 1α preparations to possess specific activities of 1.0×10^9 U/mg and 7.5×10^9 U/mg. respectively.

Human rTNFα was obtained from Biogen (Boston. MA: Batch 30. LY 18390/182. 1.5×10^7 U/mg). Protein free. phenol-water-extracted endotoxin derived from E. coli K235 (LPS) was prepared by the method of McIntire et al. (21).

All reagents were diluted to the desired concentration in pyrogen-free saline just before i.p. injection of 0.5 ml per mouse.

Measurement of CSF activity in serum. At the indicated times after injection. mice were bled from the orbital plexus. and serum was collected after clot formation by centrifugation. CSF activity was measured in pooled serum samples collected from four to five mice per treatment group per experiment. The bone marrow colony assay for CSF activity has been described in detail (22). Briefly. C3H/HeJ bone marrow cells were enriched for mononuclear cells by density gradient centrifugation on lymphocyte separation medium (Litton Bionetics. Charleston. SC). The cells collected from the interface of the gradient were washed and resuspended in RPMI 1640 supplemented with antibiotics. glutamine. sodium bicarbonate. HEPES buffer. and 15% FCS. Three serial twofold dilutions of each serum sample (30%. 15%. and 7.5% [v/v]) were prepared in this medium. and 0.2 ml of each dilution was added to each of duplicate wells in a six-well tissue culture plate. A final cell suspension was prepared at 1×10^5 cells/ml in complete medium supplemented with 0.3% Bacto-Agar (Difco. Detroit. MI) and maintained at 41°C. One milliliter per well was added immediately after resuspension of the cells in the agar-medium mixture. Once solidified. the cultures were incubated at 37°C (5% CO_2) for 6 to 7 days. at which time colonies (≥50 cells/colony) were counted under a dissecting microscope. Serum CSF activity was expressed as colony forming units (CFU) per milliliter of serum. based on colony counts within the linear part of the serial dilution curve.

In some experiments. individual colonies were extracted from the semisolid agar cultures by using either a pasteur pipette or a Pipetteman (Rainin. Woburn. MA). were resuspended in 0.2 ml FCS. and were subjected to cytocentrifugation (Cytospin 2: Shandon Southern Products. Astmoor. England). Slides were fixed in methanol and were stained with a modified Wright's stain (Diff-Quick. American Scientific Products. McGaw Park. IL).

Measurement of IFN activity in serum. The level of IFN in serum samples was measured in an antiviral assay. which tests the ability of the serum to protect murine L929 fibroblasts from infection with vesicular stomatitis virus (Indiana strain). Units of activity were based on the protection afforded by serum samples relative to the NIH International Standard (Cat. No. G-002-904-511). This assay has been described in detail (23).

RESULTS

Induction of CSF in vivo by murine and human rIL 1α. Intraperitoneal injection of mice with murine rIL 1α resulted in a time- and dose-dependent induction of serum CSF activity. Figure 1 illustrates that in response to injection of an optimal dose of murine rIL 1α (determined from Fig. 2). induction of CSF activity peaked by 3 hr and was reduced significantly by 6 hr and 24 hr postinjection.
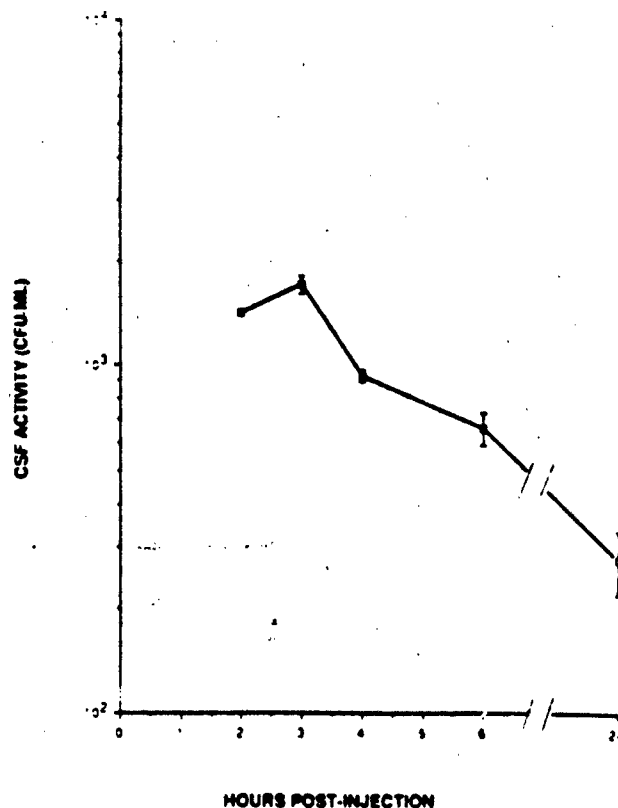

HOURS POST-INJECTION

Figure 1. Time course for the induction of CSF in vivo by murine rIL 1. C57BL/6J mice were injected i.p. with 1300 U of murine rIL 1α. At the indicated times postinjection. groups of five mice were bled: their serum was pooled and assayed for CSF activity as described in *Materials and Methods.* Pooled sera from mice injected with saline contained 0 CFU/ml. Serum from mice injected with LPS (25 μg). collected at 6 hr postinjection. contained 3260 ± 220 CFU/ml. The results represent the means ± SEM and were derived from a single representative experiment.

Neither saline nor a control bacterial extract (provided in the same Gu-HCl carrier as the rIL 1α and injected at concentrations equivalent to as much as 4000 U of the biologically active rIL 1α preparation) induced any CSF activity over a 24-hr period. Maximum rIL 1α-induced CSF activity preceded optimal induction of CSF by E. coli K235 LPS. shown previously to peak at approximately 6 hr postinjection (15). although the magnitude of the rIL 1α-induced response was usually slightly less than that seen in LPS-injected animals. Figure 2 shows a dose-response curve for the induction of CSF by murine rIL 1α. 3 hr postinjection. Maximum CSF induction by murine rIL 1α was typically observed between 7×10^2 and 2×10^3 U of activity. The murine rIL 1α had no CSF activity itself. as evidenced by a failure to induce bone marrow-derived colonies in soft agar (data not shown).

The induction of CSF in mice by human rIL 1α was very similar to that observed with murine rIL 1α with respect to both time and dose dependencies (Table I and Fig. 3). Figure 3 presents a composite of individual data points derived from 11 separate experiments. which shows that the responses induced by murine and human rIL 1α preparations peaked at approximately 3 hr postinjection and were not significantly different from each other. or from that induced by LPS. It should also be noted that murine and human rIL 1α induced CSF comparably in a variety of outbred and inbred mouse strains.
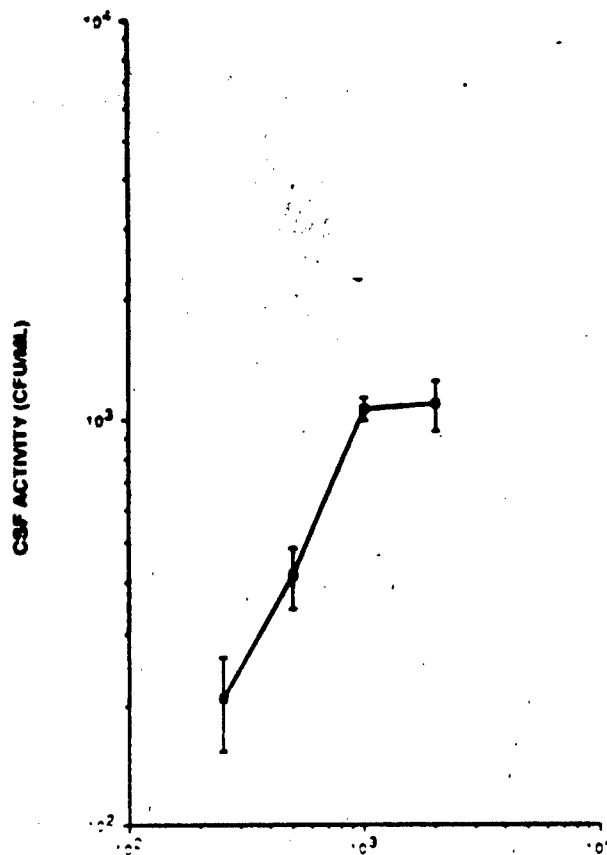
Figure 2. Dose-response curve for the induction of CSF in vivo by murine riL 1α. Groups of C57BL/6J mice (five mice per group) were injected with the indicated concentration of murine riL 1α and were bled 3 hr postinjection. The serum was pooled within a treatment group and was assayed for CSF activity as described in Materials and Methods. Serum from mice injected with saline contained 0 CFU/ml. The results represent the means ± SEM and were derived from a single representative experiment.

DOSE OF MURINE riL-1α INJECTED (UNITS)

TABLE I
Dose-dependent induction of CSF in mice injected with human recombinant IL 1α

Treatment[a]	CSF Activity (CFU/ml)
Saline	3 ± 3
7.5 × 10¹ U (10 ng)	70 ± 10
3.75 × 10² U (50 ng)	500 ± 160
7.5 × 10² U (100 ng)	2211 ± 341
3.75 × 10³ U (500 ng)	2373 ± 375
7.5 × 10³ U (1000 ng)	2490 ± 88
1.5 × 10⁴ U (2000 ng)	2750 ± 200

[a] Groups of C57BL/6 mice (four to five mice per group) were injected i p with saline or the indicated dose of human riL 1α and were bled 3 hr after injection. The results represent the means ± SEM of data pooled from three separate experiments

Including endotoxin-hyporesponsive C3H/HeJ strain (Table II). This latter finding supports the hypothesis that riL 1α, and not an endotoxin contaminant of the recombinant preparations, is responsible for induction of CSF. Analysis of cytospin preparations of individual colonies revealed that the CSF activity which was induced resulted in the development of both macrophages and granulocytes from bone marrow progenitor cells (Fig. 4a).
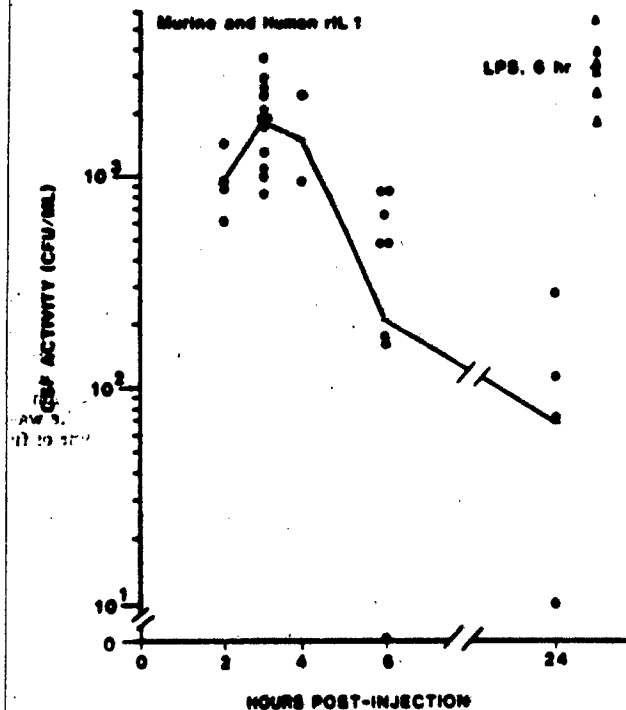

Figure 3. Composite time course for the induction of CSF in vivo by murine (●) or human (O) riL 1α (750 to 4000 U) was injected into groups of five mice (C57BL/6J, C3H/HeJ, C3H/OuJ, C3H/HeN, or HSD(ICR)BR strains). The mice were bled at the indicated times postinjection, and the pooled sera were assayed for CSF activity. The results represent a compilation of data from 11 separate experiments. Neither saline nor control bacterial extract induced >10 U/ml CSF activity in any strain or at any time point. Triangles represent the CSF response of different groups of mice to LPS (25 μg) at 6 hr postinjection. The small bar within each time point indicates the geometric mean of the individual data points for that time point.

TABLE II
Induction of CSF activity in C3H/HeJ mice by human riL 1α

Mouse Strain	Treatment[a]	CSF Activity (CFU/ml)
C57BL/6	Normal serum	0
	Human riL 1α: 3 hr	1740 ± 142
C3H/HeJ	Normal serum	10 ± 10
	Human riL 1α	
	3 hr	1817 ± 178
	6 hr	845 ± 87
	24 hr	110 ± 10

[a] Groups of C57BL/6 or C3H/HeJ mice (five mice per group) were injected with nothing (normal serum) or 750 U (100 ng) human riL 1α i p and were bled at the indicated times after injection. The results represent the means ± SEM derived from a single representative experiment

In addition to measuring CSF activity, every serum pool was also screened for the presence of IFN activity in a standard antiviral assay. Neither murine nor human riL 1α induced detectable IFN in vivo (x̄ = <2.2 ± 0.4 U/ml, n = 30). In contrast to the IFN levels induced by LPS between 2 and 6 hr postinjection (x̄ = 129 ± 34 U/ml, n = 7).

Induction of CSF in vivo by human rTNFα. Recent studies by Beutler, Cerami, and Milsark (3, 7) have implicated TNF as another important mediator of LPS-induced toxicity. We therefore examined the ability of human rTNF-α to induce CSF in vivo. Like riL 1α, rTNFα i) induced CSF (mixed granulocyte-macrophage colonies: Fig. 4b) in a time- and dose-dependent fashion (Fig. 5). ii)

Figure 4. Cytospin preparations of colonies derived from rIL 1a- (a) or rTNFa-(b) induced CSF.

failed to induce IFN activity (<1.5 ± 0.5 U/ml, n = 7). Two differences between rTNFa- and rIL 1a-induced CSF were observed. First, the concentrations of rTNFa required to induce CSF were considerably higher than the concentrations of either murine or human rIL 1a preparations. Second, by 6 hr after administration of rTNFa (Fig. 5), the CSF response had declined to a much lower level than observed at 6 hr post-rIL 1a (Fig. 1).

Table III demonstrates that combined injection of rTNFa and human rIL 1a in suboptimal concentrations resulted in additive CSF induction, whereas simultaneous injection of optimal doses of each cytokine failed to induce greater levels of CSF than elicited by either cytokine alone. Combined treatment did not induce IFN activity (<0.4 U/ml).
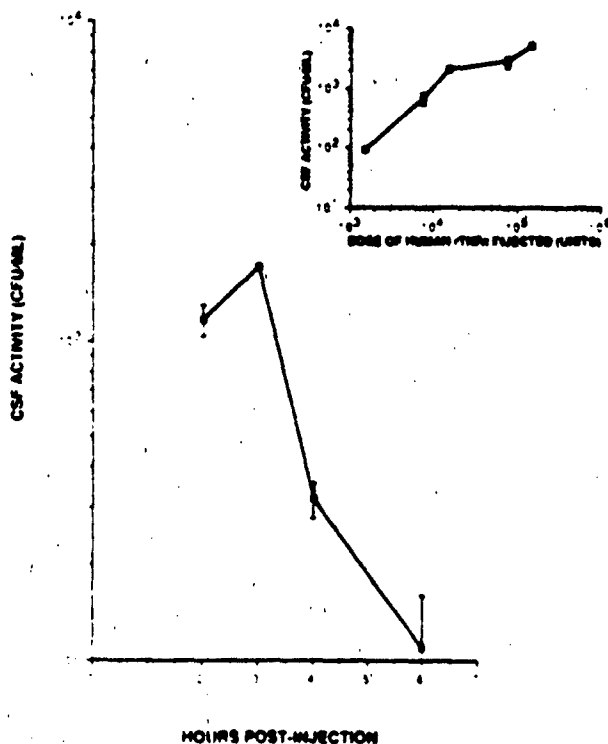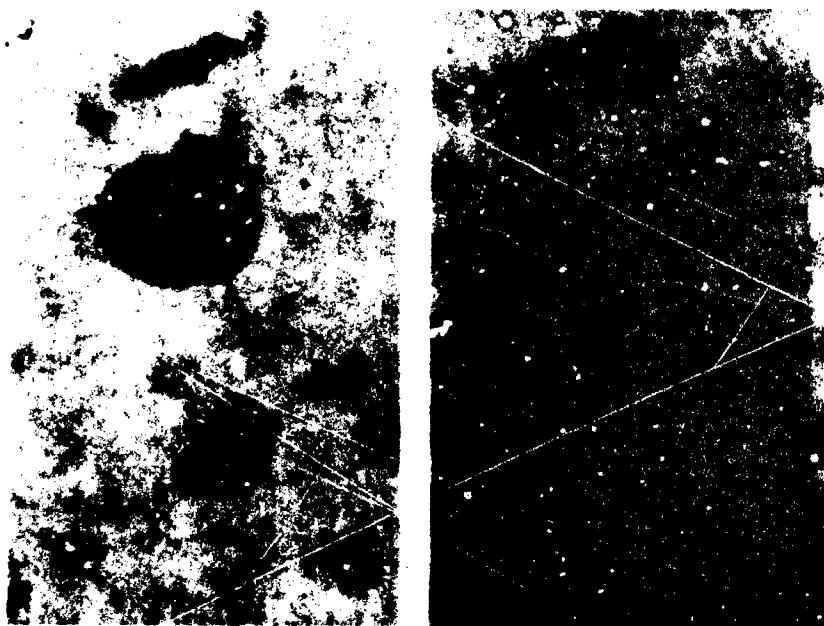
Figure 5. Time course and dose-response curves for rTNFa-induced CSF. Figure illustrates the time course for the induction of CSF by 7.5 × 10⁴ U (5 μg) rTNFa in C57BL/6 mice and represents the mean ± SEM of two separate experiments. Inset shows the dose-response curve for CSF induction by rTNFa. Based on the specific activity of this material, 1 U = 0.067 ng. Results represent the means ± SEM and were derived from four separate experiments in which C57BL/6 and C3H/HeN mice (four to five mice per treatment group per experiment) were injected. In all of these experiments, pooled normal serum or serum from saline-injected animals contained 0 CFU/ml

was active in the endotoxin-hyporesponsive C3H/HeJ mouse strain (saline-injected = 0 CFU/ml; rTNFa (5 μg)-injected = 3,530 CFU/ml, 3 hr postinjection), and iii)

TABLE III
Induction of CSF activity by combined treatment with human rIL 1a and rTNFa

Treatment[a]	CSF Activity (CFU/ml)
Saline	0
rIL 1a:	
7.5 × 10⁴ U (10 ng)	1190 ± 325
3.75 × 10⁵ U (50 ng)	1600 ± 195
7.5 × 10⁵ U (100 ng)	3620 ± 683
rTNFa:	
1.5 × 10⁵ U (100 ng)	90 ± 42
7.5 × 10⁵ U (500 ng)	516 ± 99
7.5 × 10⁴ U (5 μg)	3435 ± 383
Combined treatment:	
rIL 1a (10 ng) + rTNFa (100 ng)	993 ± 102 (1280 ± 327)[b]
rIL 1a (10 ng) + rTNFa (500 ng)	2400 ± 235 (1706 ± 339)[b]
rIL 1a (50 ng) + rTNFa (100 ng)	2305 ± 375 (1690 ± 199)[b]
rIL 1a (50 ng) + rTNFa (500 ng)	2960 ± 414 (2116 ± 218)[b]
rIL 1a (100 ng) + rTNFa (5 μg)	2900 ± 689 (7055 ± 783)[b]

[a] Groups of C57BL/6 mice (four mice per group) were injected i.p. with saline or the indicated dose of human rIL 1a and/or rTNFa and were bled 3 hr after injection. CSF activity in the serum was measured as described in Materials and Methods. Results represent the means ± SD of two separate experiments.

[b] The number in parentheses is the expected CSF activity based on the sums of the individual treatment means and their calculated combined standard deviations.

DISCUSSION

Endotoxin is a potent inflammatory agent that induces in susceptible hosts a spectrum of responses which range from being highly beneficial to being extremely toxic—i.e., increased nonspecific resistance to infection; protection against lethal irradiation; the induction of early and late phase endotoxin tolerance and adjuvanticity (24–27); and induction of fever (28), hypoglycemia (29), abortifacient effects (30), shock and death (1). It is now appreciated that many of these physiologic changes are attributable to the action of specific, LPS-induced, macrophage-derived factors on target cells in the host. For instance, IL 1 has been shown to act on the hypothalamic region of the brain to induce fever (31) and to induce synthesis of acute-phase reactants by hepatocytes (4). Glucocorticoid antagonizing factor induces hypoglycemia by blocking glucocorticoid induction of hepatic enzymes, such as phosphoenolpyruvate carboxykinase, which in turn blocks gluconeogenesis (8). More recently, a role for TNF in endotoxicity was supported by the demonstration that passively administered, anti-TNF antibodies prevented LPS-induced lethality (7). Previous studies indicated that in vivo, LPS induced the maximal appearance of both IL 1 and TNF in serum within approximately 2 hr (10–12). An unexpected relationship between IL 1 and TNF was reported recently by Dinarello et al. (13) and Bachwich et al. (14). Both groups showed that rTNF induced IL 1 in vitro. In vivo, high doses of rTNF were found to induce two temporally distinct febrile responses (13). Differences in heat stability between TNF and IL 1 and the use of highly specific anti-human IL 1 antibodies prompted Dinarello et al. (13) to conclude that 1) TNF itself was a pyrogen, and 2) TNF-induced IL 1 was responsible for the second febrile response.

Clearly, less is understood about the cellular mechanisms that underlie some of the other manifestations that follow LPS administration, and especially those that result in hematopoietic changes. For instance, LPS induces an influx of neutrophils into the blood and peritoneal cavity (16, 32), as well as an increase in macrophage progenitor cells in the bone marrow, 3 days after injection (23). Because previous work by Kampschmidt, Upchurch, and Pullam (16, 17) showed that partially purified preparations of "leukocyte endogenous mediator" (which contained IL 1 and probably TNF) induced CSF activity in vivo within 6 hr, we sought to confirm and extend these findings by using recombinant preparations of IL 1α and TNFα. The results presented herein demonstrate that i.p. injection of murine and human rIL 1α preparations induced in mice CSF activity that peaked by 3 hr post-administration. On the basis of activity in the thymocyte proliferation assay, the murine and human rIL 1α preparations induced CSF over the same concentration range and were both as active in endotoxin-hyporesponsive C3H/HeJ mice as in normally endotoxin-responsive strains. Recombinant TNFα also induced CSF activity when administered i.p., but on a concentration basis, was considerably less efficacious than rIL 1α, and the response was less sustained over time than that induced by rIL 1α. However, these dose differences may be related to the route of administration chosen for use in these studies. For both human rIL 1α and rTNFα, low levels of serum CSF activity were detectable as early as 1 hr postinjection (data not shown). Because rTNFα induced levels of CSF that were comparable to those induced by rIL 1α, the possibilities exist that 1) TNF can act independently of IL 1 as an inducer of CSF and/or 2) rTNF induces the CSF activity via an IL-1 intermediate. Even though the time courses for the induction of CSF by rTNFα and rIL 1α were similar, (i.e., we did not see any indication of a second, later peak of CSF in response to rTNF), and the simultaneous injection of optimal doses of two cytokines were not additive for induction of CSF, we cannot at this time dismiss the possibility that at very high concentrations of rTNFα (>10 μg) a second, IL 1-induced CSF peak might manifest itself.

The exact molecular species of CSF induced by rIL 1α or rTNF in vivo is not known at this time. Clearly, both induced an activity in the serum that resulted in primarily mixed granulocyte-macrophage colonies (Fig. 4). However, this could be due to a mixture of CSF species (e.g., a granulocyte-specific CSF [G-CSF] plus macrophage-specific CSF-1, and/or to the activity of a single GM-CSF species). Results of early work using various affinity purification techniques to characterize the CSF activity in serum derived from LPS-injected mice suggested that a mixture of CSF species were produced in response to LPS (33).

The finding that neither rIL 1α nor rTNF induced detectable levels of IFN in the serum was surprising in light of several recent reports that have indicated that both cytokines induce IFN in vitro in fibroblast cultures (34, 35). LPS has been reported to induce IFN activity in serum, which, like IL 1 and TNF, peaks at 2 hr (9). Studies indicate that LPS-induced IFN is produced principally by macrophages (36), and would therefore presumably be a form of IFN-α, although this has not been confirmed with the use of antibodies specific for IFN-α (37). Thus, differences between in vivo and in vitro cytokine-induced IFN production may be related to differences in "target cells."

The induction of CSF activity by LPS (15, 38) may well be the direct effect of LPS on certain cell types (e.g., macrophages or endothelial cells). In a very recent report, Munker et al. (39) demonstrated that TNF can act directly in vitro to induce production of GM-CSF in normal lung fibroblasts and vascular endothelial cells, as well as in certain malignant cell types. However, the data presented herein suggest several alternative pathways by which CSF production might be stimulated indirectly following LPS administration (i.e., LPS → IL 1 → CSF; LPS → TNF → CSF; LPS → TNF → IL 1 → CSF). Future experiments in which IL 1-specific antagonists (such as monoclonal or polyclonal anti-murine IL 1 antibodies) are administered in vivo in combination with LPS or rTNFα should enable a delineation of these pathways and their actual dependancy on IL 1 as an intermediate in this inflammatory cascade.

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